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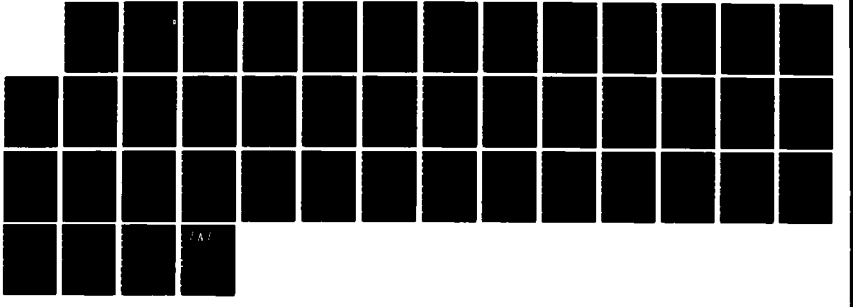

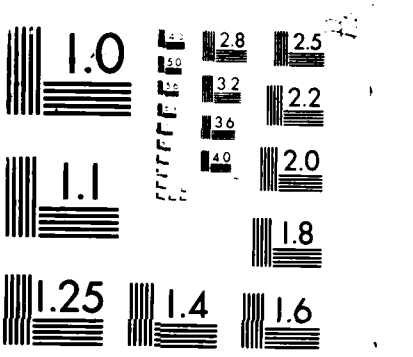

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DEVELOPMENT OF SYSTEMS FOR THE DELIVERY OF ANTIVIRAL DRUGS

ANNUAL REPORT

William M. Shannon
John A. Secrist III
Patricia E. Noker
Lucy M. Rose

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October 31, 1986
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This is our First Annual Progress Report on Contract No. DAMD17-85-C-5276. It covers research performed during the period from September 30, 1985 through September 29, 1986.

Ribavirin, a broad-spectrum antiviral agent with potent activity in vitro against a number of important RNA viruses of military significance, is severely limited in its usefulness against virus-induced encephalitic diseases because it does not cross the blood-brain barrier well enough to achieve effective antiviral concentrations in the brain. Our efforts are directed toward the brain-specific delivery of ribavirin and other antiviral agents by means of a redox prodrug concept. The scope of the research program involves the synthesis of CNS-targeted prodrug esters of ribavirin and selenazole, pharmacokinetic studies of drug distribution and sustained delivery of drug in the brain, and the evaluation of the therapeutic efficacy of these antiviral prodrugs compared with the parent drugs in the treatment of lethal VEE and JE infections in mice. We have

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synthesized the first of these prodrugs by a five route starting from ribavirin. In preliminary studies, this prodrug has protected mice from a lethal challenge of JE virus and was much superior in efficacy to the parent drug, which had no effect. Extracture and HPLC assay procedures necessary for the proposed pharmacokinetic studies with the ribavirin prodrug in mice have been developed.

DEVELOPMENT OF SYSTEMS FOR DELIVERY
OF ANTIVIRAL DRUGS

SUMMARY

This is our First Annual Progress Report on Contract No. DAMD17-85-C-5276. It covers research performed during the period from September 30, 1985 through September 29, 1986.

Ribavirin, a broad-spectrum antiviral agent with potent activity in vitro against a number of important RNA viruses of military significance, is severely limited in its usefulness against virus-induced encephalitic diseases because it does not cross the blood-brain barrier well enough to achieve effective antiviral concentrations in the brain. Our efforts are directed toward the brain-specific delivery of ribavirin and other antiviral agents by means of a redox prodrug concept. The scope of the research program involves the synthesis of CNS-targeted prodrug esters of ribavirin and selenazole, pharmacokinetic, studies of drug distribution and sustained delivery of drug in the brain, and the evaluation of the therapeutic efficacy of these antiviral prodrugs compared with the parent drugs in the treatment of lethal Venezuelan equine encephalitis (VEE) virus and Japanese encephalitis (JE) virus infections in mice. We have synthesized the first of these prodrugs by a five-step route starting from ribavirin. In preliminary studies, this prodrug has protected mice from a lethal challenge of JE virus and was much superior in efficacy to the parent drug, which had no effect. Extraction and HPLC assay procedures necessary for the proposed pharmacokinetic studies with the ribavirin prodrug in mice have been developed.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been found to possess broad-spectrum antiviral activity both in vitro and in vivo (1-5). Studies conducted at Fort Detrick have clearly demonstrated that ribavirin is markedly effective against bunyaviruses (e.g., Rift Valley fever virus) and arenaviruses (e.g., Lassa fever virus, Pichinde virus, and Machupo virus), but that it has only minimal to no efficacy against the alphaviruses (e.g., Venezuelan equine encephalitis virus and Chikungunya virus) or flaviviruses (e.g., Japanese encephalitis virus and yellow fever virus) in vivo. The apparent inability of ribavirin to achieve effective antiviral concentrations in the brain and central nervous system significantly limits its usefulness against those viruses which cause primary encephalitis (6). Ribavirin also does not prevent the late encephalitic phase of the diseases caused by Rift Valley fever, Junin, and Machupo viruses (7). The principal reason for this lack of efficacy is the relative inability of the drug to cross the blood-brain barrier and to concentrate in the central nervous system.

A related compound (2-β-D-ribofuranosylselenazole-4-carboxamide; selenazole) has been synthesized by Srivastava and Robins (8) and has been shown to exhibit potent, broad-spectrum in vitro activity against selected DNA-containing and RNA-containing viruses (9), including many viruses of potential military importance. Selenazole appears to be significantly more potent than ribavirin against paramyxoviruses, reoviruses, togaviruses, bunyaviruses, arenaviruses, picornaviruses, rhabdoviruses, herpesviruses, and pox virus in vitro. It is extremely active against yellow fever virus and is a prime candidate for antiviral chemotherapy studies in vivo. Another compound, tiazofurin, has been found to exert in vitro activity against flaviviruses and Korean hemorrhagic fever (KHF) virus. Both selenazole and tiazofurin appear to be rapidly excreted in vivo, so that a prodrug form of these drugs would be desirable to develop.

When selenazole is used in combination with ribavirin in vitro, synergistic antiviral effects are observed against Venezuelan equine encephalitis (VEE) virus and Japanese encephalitis (JE) virus (10). Synergistic activity has been shown for tiazofurin in combination with ribavirin against yellow fever virus in vitro, but not for other viruses. These observations indicate that combination antiviral chemotherapy with these agents and ribavirin might be a useful approach to the treatment of flavivirus infections in vivo.

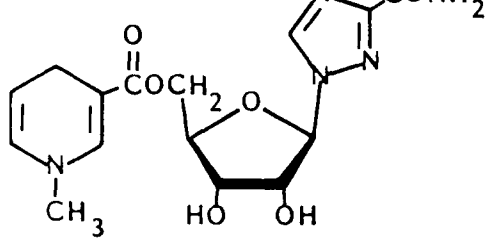
Since many of the target viruses of interest to the Army produce a lethal encephalitis in the host, we believe that efforts directed toward the brain-specific delivery of candidate antiviral drugs will be an important approach to improving the efficacy of such drugs against agents of military significance. We, therefore, plan to

develop systems for the effective delivery of antiviral drugs across the blood-brain barrier.

Our initial efforts have been directed toward the synthesis of ribavirin prodrugs. Based upon the brain-specific delivery of, for example, phenethylamine (11), dopamine (12), trifluorothymidine (13), and acyclovir (14), we expect prodrug esters of ribavirin to effectively cross the blood-brain barrier. At that point, the dihydropyridine moiety will be oxidized to the pyridinium salt which will be retained by the brain. Cleavage of the ester enzymatically will produce a sustained delivery of ribavirin in the brain. For any specific compound, the rates of the various reactions in the process must be favorable, but the success achieved in several systems thus far certainly gave credence to our proposed application of this redox delivery system approach to the sustained, site-specific administration of antiviral agents such as ribavirin.

II. CHEMISTRY

Our efforts during the first year of the contract have been expended in two directions. First, we have developed a synthesis of the ribavirin prodrug 1, starting from ribavirin. This prodrug, of course, is expected to be transported across the blood brain barrier, and then rapidly oxidized from the dihydropyridine to the pyridinium salt, which will be trapped in the brain. Esterase hydrolysis of the prodrug would then gradually free ribavirin. The hope is that enough oxidized prodrug will be trapped in the brain to allow release of a therapeutically useful concentration of ribavirin. The synthesis of 1 will be described. Our second effort has been directed toward a slightly different prodrug of ribavirin, and that research will be covered later in this section.



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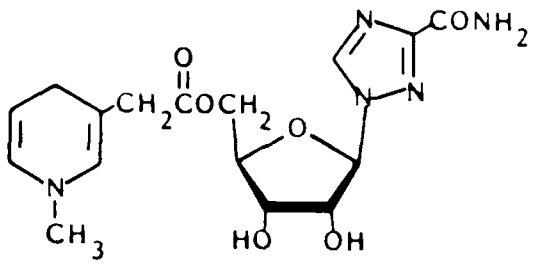
The synthetic steps leading to 1 are depicted in Scheme II-1. In order to cleanly form a 5'-nicotinate ester of ribavirin, the 2'- and 3'-hydroxyl groups must be suitably protected. An isopropylidene group, which is acid-labile, appeared to be appropriate, and the 2',3'-O-isopropylidene derivative of ribavirin (3) is a known compound (1). Preparation

of 3 was accomplished by the literature procedure, but using a flash chromatography purification. Coupling of 3 to nicotinic acid was achieved utilizing dicyclohexylcarbodiimide to produce the ester 4. Methylation of 4 on the pyridine nitrogen was accomplished with iodomethane in boiling acetone to give 5. That methylation did not occur on the triazole ring was clear from the mass spectral fragmentation pattern. Removal of the isopropylidene group from 5 occurred readily with 1N H_2SO_4 at room temperature to afford 6. An alternative synthesis of 6 was attempted by first removing the isopropylidene group from 4, producing 7, followed by methylation. This approach proved to be unsatisfactory because the methylation step did not proceed cleanly. Reduction of 6 to the target prodrug 1 was achievable with sodium dithionite under carefully controlled conditions. The reaction is best followed by HPLC, because some oxidation of 1 back to 6 occurs on a standard thin-layer plate. Proof of this oxidation on the plate was obtained by the two-dimensional development of a plate spotted with 1. Preliminary stability evaluations on 1 were carried out by HPLC analysis. In distilled water, pH 5.0-5.5, 1 had a half-life of over twenty hours. In pH 7 buffer (ammonium dihydrogen phosphate), however, the half-life for 1 was only about an hour. We have sent nine vials of 1 containing a total of 243 mg to Dr. Ussery for use in the experiment described in the Virology section of this report. In addition, we have about 400 mg of 1 (under argon in the freezer) and 4.5 g of 6 on hand for future experiments.

A potential problem with 1 is that once it is trapped in the brain, it may not release ribavirin as rapidly as desired. This process would depend upon the facility of ester cleavage. The aromatic ring directly adjacent to the ester linkage slows down the cleavage for steric reasons. Bulk on either side of the ester linkage will have such an effect. If the desired pharmacokinetics are to be obtained by speeding up esterase cleavage, then reducing the bulk on the pyridine side of the ester linkage should have the appropriate effect. A reduction in the steric bulk in the immediate area should be readily accomplished simply by adding a methylene unit. We therefore began efforts to synthesize the prodrug 8. The route by which we will prepare 8 is shown in Scheme II-2. The isopropylidene derivative of ribavirin (3) is coupled with 3-pyridylacetic acid in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine to produce ester 9. Quaternization with iodomethane was carried out following the procedure employed for 5. We now have 1.85 g of 10 accumulated for carrying on to 8, and we are currently exploring the removal of the isopropylidene group from 10.



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A. Future Plans

We will be completing the synthesis of 8 very soon, and then comparisons of 1 and 8 can be made to determine which is the superior prodrug. That infor nation will allow us to propose modified prodrugs with superior properties, if it appears necessary to do so. We will be synthesizing larger quantities of any necessary prodrugs for animal studies. We may also look at the synthesis of comparable prodrugs of other appropriate antiviral agents. Lastly, we will synthesize radiolabelled prodrugs as necessary for pharmaco logical studies. Specifically, we have obtained 0.5 mCi of ^{14}C-labelled ribavirin to be converted to ^{14}C-1. If our yields for the synthesis of the radiolabelled material are comparable to those we have obtained during the development of the route, we should obtain about 150 μCi of the labelled prodrug. This quantity should be sufficient for initial pharmacological evaluation.

B. Experimental Section

1-(2,3-O-Methylethylidene-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (3). A suspension of ribavirin (3.00 g, 12.3 mmol) in 2,2-dimethoxypropane (20 mL) and dry acetone (40 mL) was cooled to 0 °C, and 70% perchloric acid (0.6 mL) was then added. The mixture was allowed to warm to room temperature while stirring for 3 h. Tlc (9:1 $CHCl_3$/MeOH) showed that all of the starting material was gone, with the production of one major product plus a faster-traveling minor by-product. The solution was neutralized with 1 N NaOH and evaporated to dryness in vacuo. The residue was triturated with methanol (20 mL) and filtered. The filtrate was concentrated and the product crystallized from CH_3OH/EtOAc. The faster-traveling impurity was still present, so the mixture was purified by flash chromatography (9:1 EtOAc/CH_3OH). Precipitation from methanol with ethyl acetate gave 2.98 g (10.5 mmol, 85%), homogeneous by tlc, m.p. 152-54 °C (lit. m.p. 153-54 °C). The compound was used directly for further reactions.

1-[2,3-O-Methylethylidene-5-O-(3-pyridinylcarbonyl)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (4). A mixture of 2',3'-isopropylideneribavirin (2, 1.00 g, 3.52 mmol, ref. 1), nicotinic acid (435 mg, 3.54 mmol), and 4-dimethylaminopyridine (43 mg, 0.35 mmol) were stirred in THF (25 mL) at room temperature. Dicyclohexylcarbodiimide (740 mg, 3.6 mmol) was then added in one portion, and stirring continued for 1 h. The suspended solid was filtered off and washed thoroughly with ethyl acetate. The combined filtrate and washings were concentrated in vacuo and purified by flash chromatography (95:5 EtOAc/EtOH). The collected fractions gave a white foam, homogeneous by tlc, 1.09 g (2.80 mmol, 80%). An analytical sample was crystallized from a small amount of water, m.p. 92-95 °C (dec). FABMS m/z 390 $(M + 1)^+$. ^{1}H-NMR (Me_2SO-d_6) δ 9.00 (d, 1, 2"-CH), 8.81 (m, 1, 5-CH), 8.79 (d, 1, H-6"), 8.20 (dt, 1, H-4"), 7.81 and 7.64 (2 s, 2, $CONH_2$), 7.54 (m, 1, H-5"), 6.34 (dt, 1, H-1'), 5.29 (d, 1, H-2'), 5.17 (dd, 1, H-3'), 4.64 (m, 1, H-4'), 4.53-4.38 (m, 2, 5'-CH_2), 1.52 and 1.34 (2 s, 6, $-C(CH_3)_2$). Anal. Calcd for $C_{17}H_{19}N_5O_6 \cdot H_2O$: C, 50.12; H, 5.19; N, 17.19. Found: C, 49.83; H, 5.00; N, 17.54.

1-[2,3-O-Methylethylidene-5-O-(N-methylpyridinium-3-ylcarbonyl)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide Iodide (5). A solution of 4 (100 mg, 0.26 mmol) and methyl iodide (65 μL, 1.05 mmol) in acetone (10 mL) was refluxed (70 °C oil bath) for 20 h, employing an efficient condenser. The solution was cooled, and the product precipitated with ether and collected. Recrystallization from acetone/ether gave 105 mg (0.198 mmol), 76%) as a yellow solid. FABMS m/z 404 (M^+ of cation). ^{1}H-NMR (Me_2SO-d_6) δ 9.48 (s, 1, H-2"), 9.18 (d, 1, H-6"), 8.95 (d, 1, H-4"), 8.83 (s, 1, H-5), 8.24 (m, 1, H-4"), 7.84 and 7.62 (2 s, 2, $CONH_2$), 6.33 (d, 1, H-1'), 5.31 (dd, 1, H-2'), 5.21 (dd, 1, H-3'), 4.66 (m, 2, H-4' and H-5a'), 4.52 (dd, 1, H-5b'), 4.44 (s, 3, NCH_3), 1.56 and 1.37 (2 s, 6, $C(CH_3)_2$).

1-(5-O-(Pyridinylcarbonyl)-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide, Sulfate Salt (7). A solution of 4 (900 mg, 2.31 mmol) was stirred at room temperature for 20 h in 1 N H_2SO_4 (10 mL). The solution was then carefully neutralized to pH 7.5 with 1 N NaOH. This mixture was applied directly to a Bio-Bead (SM-4) column, containing 15 g of the beads as supplied. After washing the column with H_2O (100 mL), the product was eluted with 15% aqueous ethanol. After evaporating the eluant to dryness in vacuo, the white solid was recrystallized from ethanol, giving 645 mg (1.85 mmol, 80%), m.p. 180-181 °C, softening from 165 °C. FABMS m/z 350 $(M + 1)^+$. ^{1}H-NMR (Me_2SO-d_6) δ 9.05 (d, 1, H-2"), 8.86 (s, 1, H-5), 8.83 (dd, 1, H-6"), 8.37 (dt, 1, H-4"), 7.84 and 7.67 (2 s, 2, $CONH_2$), 5.97 (d, 1, H-1'), 5.72 (d, 1, 2'-OH), 5.45 (d, 1, 3'-OH), 4.60-4.39 (m, 4, H-2', H-3', 5'-CH_2), 4.30 (m, 1, H-4'). Anal. Calcd for $C_{14}H_{15}N_5O_6 \cdot 0.1C_2H_6O$: C, 48.02; H, 4.44; N, 19.79. Found: C, 47.94; H, 4.72; N, 19.95.

1-[5-O-(N-Methylpyridinium-3-ylcarbonyl)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide Sulfate Salt (6). A solution of 5 (2.25 g; 4.23 mmol) in 1 N H_2SO_4 (20 mL) was stirred at room temperature for 18 h. The volume was reduced to one half on a rotary evaporator, and 200 mL of EtOH was added. Storage in the freezer for two days yielded a pale yellow solid, 1.88 g (4.08 mmol; 96%), m.p. 177-179°C (dec). FABMS: m/z 364 (M^+). ^{1}H-NMR (Me_2SO-d_6) δ 9.50 (s, 1, H-2"), 9.21 (d, 1, H-6"), 9.05 (d, 1, H-4"), 8.88 (s, 1, H-5), 8.26 (dd, 1, H-5"), 7.88 and 7.64 (2 s, 2, $CONH_2$), 5.98 (d, 1, H-1'), 4.76-4.48 (m, 3, H-3' and H-5'), 4.46 (s, 3, N-CH_3), 4.42 (m, 1, H-2'), 4.32 (m, 1, H-4'). Anal. Calcd for $C_{15}H_{18}N_5O_6^+ \cdot HSO_4^-$: C, 39.05; H, 4.15, N, 15.18; S, 6.95. Found: C, 38.90; H, 4.18; N, 15.02; S, 6.88.

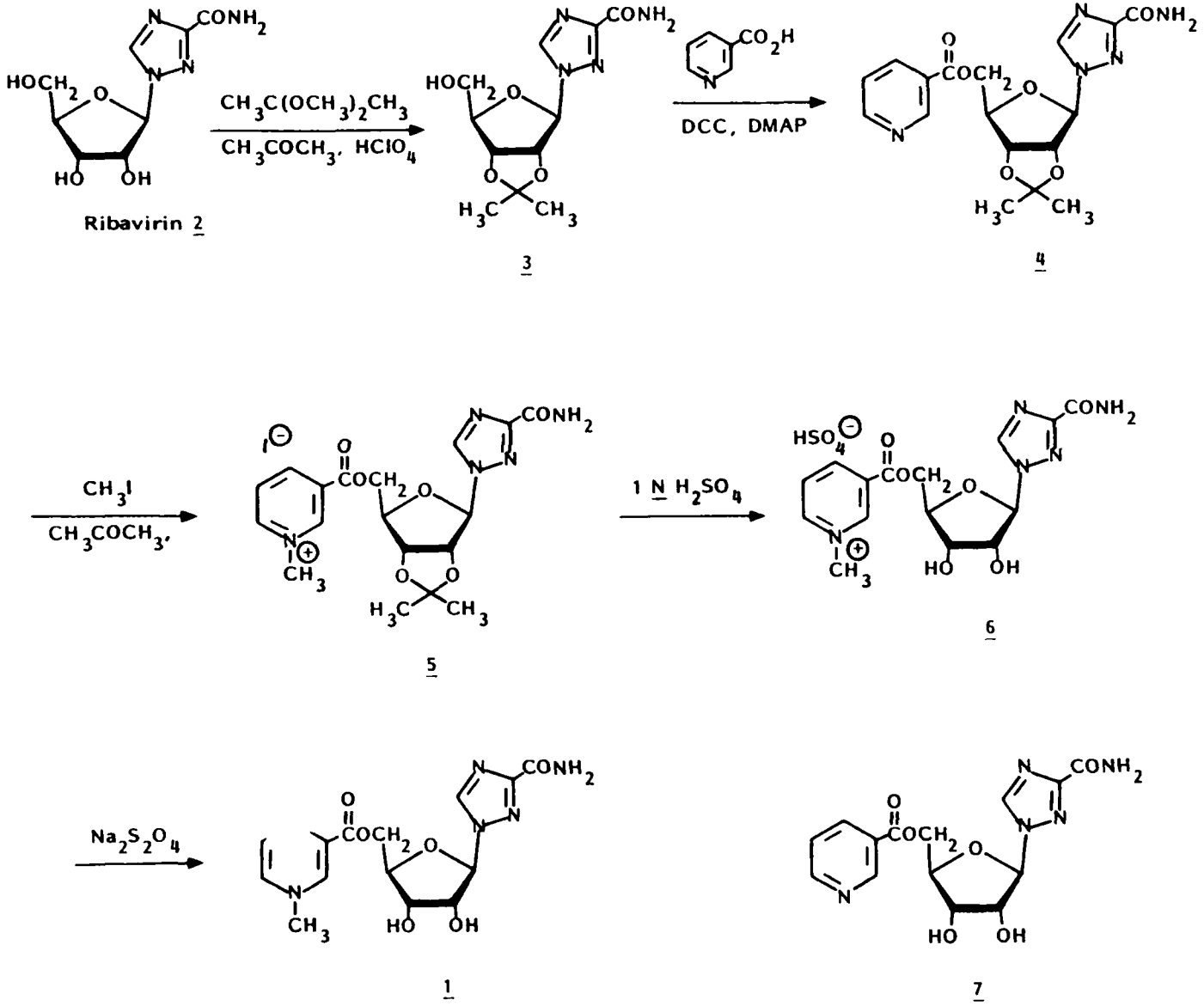
1-[5-O-(N-methyl-1,4-dihydropyridinylcarbonyl)-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide Monohydrate (1·H_2O). A solution of 6 (613 mg, 1.33 mmol) and sodium bicarbonate (672 mg; 8 mmol) in 10 mL of deoxygenated H_2O was cooled in an ice bath under a nitrogen atmosphere. Sodium dithionite (820 mg of Aldrich 85% technical grade; 4.0 mmol) was added; after 1 min a precipitate began forming. After 5 min. the solid was collected under N_2 and washed thoroughly with deoxygenated H_2O, then quickly transferred and dried under high vacuum at room temperature. The filtrate was applied to a Bio-Bead SM-4 column (1 x 10 cm) and washed with 20 mL H_2O. The Bio-Bead column we prepared by washing first with degassed H_2O, then degassed CH_3OH, then back flushing with degassed water to remove air bubbles, and then thoroughly washing with H_2O. Methanol (50 mL) was then used to elute the product. The solution was evaporated nearly to dryness in vacuo; addition of H_2O caused a precipitate which was treated as before. The first crop weighed 350 mg; the second 48 mg (total yield 1.04 mmol, 78%). Both crops were identical by melting point and NMR. M.p. 111-113°C (dec). FABMS: m/z 366 (M + 1)$^+$. ^{1}H-NMR (Me_2SO-d_6) δ 8.81 (s, 1, H-5), 7.81 and 7.63 (2 s, 2, $CONH_2$), 7.00 (d, 1, H-2"; $J_{2",4"}$ = 1.5 Hz), 5.88 (d, 1, H-1'), 5.82 (complex m, 1, H-6"; $J_{1",6"}$ = 1.5 Hz, $J_{4",6"}$ = 1.6 Hz, $J_{5",6"}$ = 8.0 Hz), 5.64 (d, 1, 2'-OH), 5.35 (d, 1, 3'-OH), 4.71 (dt, 1, H-5"; $J_{4",5"}$ = 3.4 Hz), 4.13 (m, 2, H-5'), 2.94 (s, 3, N-CH_3), 2.93 (m, 2, H-4", seen as shoulder of N-CH_3 peak). ^{13}C-NMR (Me_2SO-d_6) δ 166.65 ($CONH_2$), 160.19 (C-5"), 157.43 (CO_2), 145.27 (C-5), 142.85 (C-2"), 129.76 (C-6"), 103.60 (C-5"), 94.38 (C-3"), 91.26 (C-1'), 81.94 (C-4'), 74.04 (C-3'), 70.27 (C-2'), 62.84 (C-5'), 40.05 (N-CH_3), 21.52 (C-4"). Anal. Calcd for $C_{15}H_{19}N_5O_6 \cdot H_2O$: C, 47.00; H, 5.52; N, 18.27. Found: 46.90; H, 5.55; N, 18.18. UV (H_2O) λ_{max} 367 (7500).

1-[2,3-O-Methylethylidene-5-O-[3-(pyridylmethyl)carbonyl]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (9). A mixture of 3 (2.97 g, 10.45 mmol), (3-pyridyl)-acetic acid hydrochloride (2.00 g, 11.50 mmol), and 4-dimethylaminopyridine (0.43 g, 3.50 mmol) was stirred in dry THF (100 mL) at room temperature. Dicyclohexylcarbodiimide

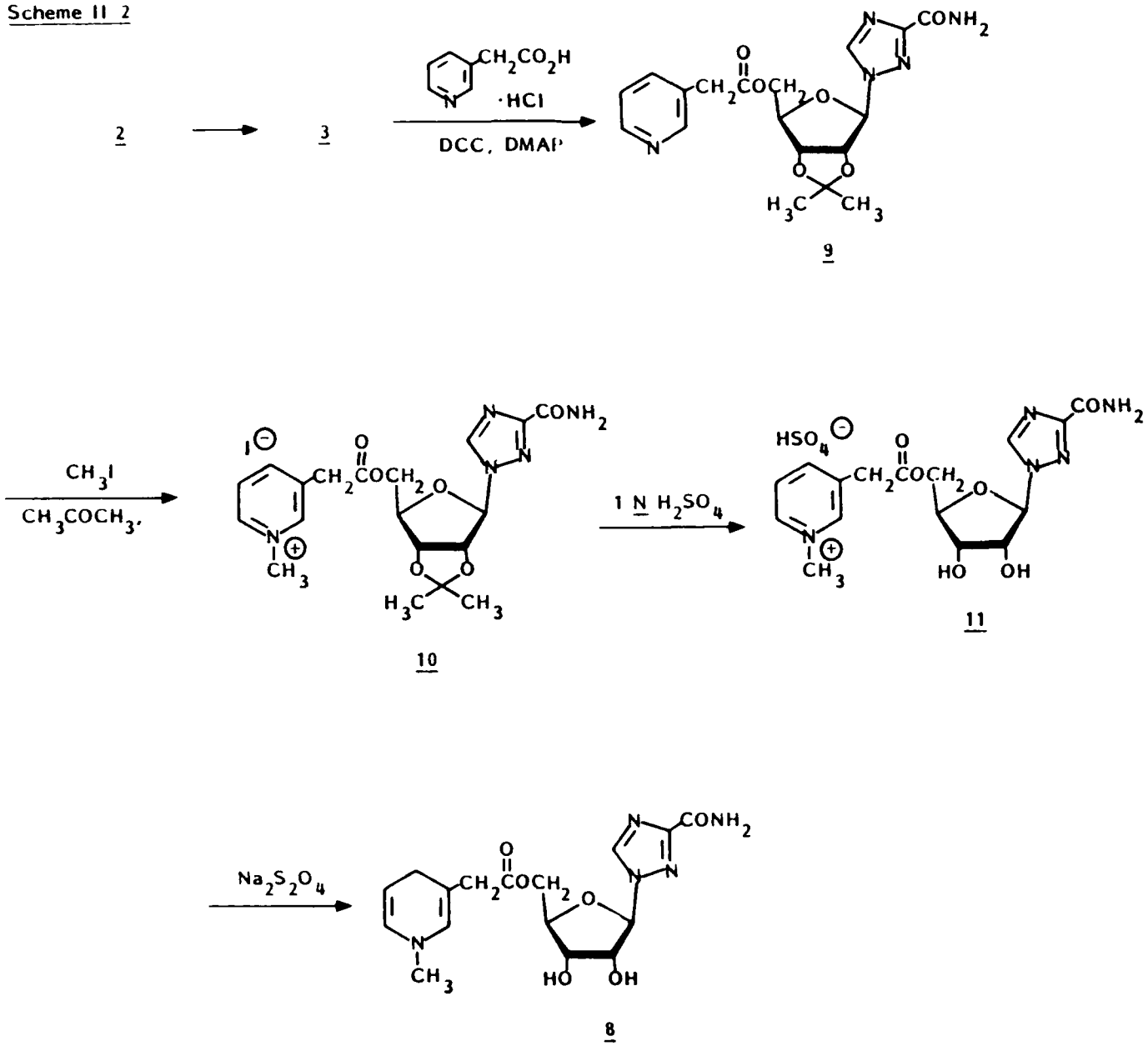
(4.30 g, 20.88 mmol) was added and stirring continued for two days. The suspended solid was filtered and washed with ethyl acetate. The combined filtrates were concentrated under reduced pressure and purified by flash chromatography (95:5 $CHCl_3$/MeOH). The homogeneous fractions were combined and dried (Na_2SO_4) to give 2.76 g (65%) of a white foam, which was taken up in $CHCl_3$ and precipitated with petroleum ether. This tacky material was suitable for use in the next step. FABMS m/z 404 $(M + 1)^+$. ^1H NMR ($CDCl_3$) δ 8.53 (dd, 1, H-6"), 8.51 (d, 1, H-2"), 8.31 (s, 1, H-5), 7.62 (dt, 1, H-4"), 7.29 (dd, 1, H-5"), 7.25 and 6.46 (2 s, 2, $CONH_2$), 6.08 (d, 1, H-1'), 5.33 (dd, 1, H-2'), 4.89 (dd, 1, H-3'), 4.55 (m, 1, H-4'), 4.29 (dd, 1, H-5a'), 4.21 (dd, 1, H-5b'), 3.63 (s, 2, $-C\underline{H}_2\overset{O}{\overset{\|}{C}}O$), 2.27 (impurity ? exchanges with D_2O), 1.58 and 1.36 (2 s, 6, $C(CH_3)_2$).

1-[2,3-O-Methylethylidene-5-O-[N-methyl[(3-pyridiniumylmethyl)carbonyl]]-β-D-ribofuranosyl]-1H-1,2,4-triazole-3-carboxamide (10). A solution of 9 (2.49 g, 6.17 mmol) and iodomethane (1.43 mL, 22.7 mmol) in 90 mL of acetone was refluxed in a 70 °C oil bath. After 5 h an orange/yellow precipitate (gummy residue) had formed, from which the yellow solution was decanted. This solution was concentrated in vacuo to 3/4 of the original volume and the product was precipitated with 200 mL of ether. The product was collected by filtration, rinsed with ether and dried in vacuo to give a light, yellow powder, 1.43 g. The orange/yellow residue was collected as an orange foam, 1.32 g, which was identical to the light yellow precipitate by mass spectral analysis. Total yield, 2.75 g (82%). FABMS m/z 418 (M^+ of cation). ^1H NMR (Me_2SO-d_6) δ 8.96 (s, 1, H-2"), 8.93 (d, 1, H-6"), 8.86 (s, 1, H-5), 8.50 (d, 1, H-4"), 8.12 (dd, 1, H-5"), 7.94 and 7.72 (2 s, 2, $CONH_2$), 6.35 (d, 1, H-1'), 5.21 (dd, 1, H-2'), 5.04 (dd, 1, H-3'), 4.48 (m, 1, H-5a'), 4.35 (s, 3, $\overset{\oplus}{N}CH_3$), 4.31 (d, 1, H-4'), 4.17 (dd, 1, H-5b'), 4.04 (s, 2, $-C\underline{H}_2\overset{O}{\overset{\|}{C}}O-$), 1.52 and 1.33 (2 s, 6, $C(CH_3)_2$). Anal. Calcd for $C_{19}H_{24}IN_5O_6$: C, 41.84; H, 4.44; N, 12.84. Found: C, 41.44; H, 4.57; N, 12.43.

Scheme II-1


CONH2
HOCH2
O
HO
OH
Ribavirin 2
CH3C(OCH3)2CH3
CH3COCH3, HClO4
3
CO2H
DCC, DMAP
COCH2
4
CH3I
CH3COCH3,
5
1 N H2SO4
HSO4
6
Na2S2O4
1
7

Scheme II 2



III. PHARMACOLOGY AND HPLC ASSAY PROCEDURES

A. HPLC Assays

1. Ion-Exchange (Anion) for Ribavirin and Its Nucleotides

Ribavirin is retained by reverse-phase HPLC columns with difficulty, whereas both the oxidized and reduced forms of its prodrug derivative require high percentages of an organic modifier for elution. It was highly unlikely, therefore, that a single HPLC assay could be readily developed for the concomitant assay of all three possible forms of this antiviral agent present in a given sample. In addition to this problem, each of the compounds exhibited a different UV maximum. For this reason, we approached assay development from two directions. One assay was developed for ribavirin alone and a second assay was developed by which both forms of the prodrug could be assayed.

A procedure previously developed in our laboratory (15) was applicable to the study of the metabolism of [3-^{14}C] ribavirin to its nucleotides. Using this procedure, sample extracts were chromatographed on a Partisil-10 SAX anion exchange column at ambient temperature. A linear gradient (50 min) from 5mM $NH_4H_2PO_4$(pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7) was used with a flow rate of 2 ml/min. Fractions were collected directly from the column eluate into vials and radioactivity for each fraction was determined (Figures III-1 and III-2).

2. Reverse-Phase HPLC

a. Ribavirin

For the ribavirin assay, a Spherisorb ODS (5μ) column (5% carbon load) was used with water as the mobile phase. The flow rate was 1 ml/min and the column eluate was monitored by UV at 254 nm (Figure III-3). In this system ribavirin was retained between 5-6 min. When the column eluate was monitored at 207 nm (the UV maximum of ribavirin), sensitivity was increased by as much as 50-fold (Figure III-4).

b. Ribavirin Prodrug: Oxidized and Reduced Forms

In the assay for the oxidized and reduced forms of the prodrug, the Spherisorb ODS (5μ) column (5% carbon load) was used with 25 mM $NH_4H_2PO_4$ (pH 4.5):CH_3CN(85:15; V,V) as the mobile phase. The flow rate was 0.8 ml/min and the column eluate was monitored by UV at 254 nm for the oxidized form and at 340 nm for the reduced form of the prodrug (Figure III-5).

c. Ribavirin and Both the Oxidized and Reduced Forms of the Ribavirin Prodrug

With these assays in place we then investigated the possibility of developing a single assay for all three compounds. Using a Whatman ODS-2 reverse-phase column (15% carbon load) with water as the mobile phase, the retention time for ribavirin increased from 5-6 min to 10 minutes, but quantitation was unreliable because of peak asymmetry. These factors (increased retention time and peak asymmetry) indicated that gradient elution in conjunction with this column might offer a solution to the problem of a single assay.

Using a 20 min linear gradient from 25 mM $NH_4H_2PO_4$(pH 4.5) to 25 mM $NH_4H_2PO_4$ (pH 4.5):CH_3CN(87.5:12.5; V,V) we achieved baseline resolution of the three compounds with ribavirin at 6.2 min, oxidized ribavirin prodrug at 13.4 min and reduced ribavirin prodrug at 24.6 min (Figure III-6). Attempts will be made to optimize this assay before it is challenged with sample extracts.

B. Extraction Procedures and Stability Studies

1. Ribavirin and Its Nucleotides from Cells

In developing acceptable extraction procedures, our criteria were that (a) they have a reproducible recovery efficiency of at least 85%, (b) that the extracts produced would not contain components which would interfere with the detection and quantitation of the compounds of interest, and (c) that the inhibitors and their metabolites be stable in the extraction matrix for at least 24 hrs at room temperature.

For the extraction of ribavirin and its nucleotides a procedure previously developed in our laboratory was applicable (15). The sample was extracted with cold 0.5N perchloric acid (PCA) and the precipitated proteins were removed by centrifugation. The pH of the supernatnant was adjusted to 7.5 with potassium bicarbonate and the precipitated $KCLO_4$ was removed by centrifugation. The supernatant was concentrated to dryness by lyophilization and the residue was dissolved in water for HPLC analysis. Assay of samples supplemented with radioactive nucleotides indicate that extraction efficiency for this procedure is greater than 90%. These samples must be kept cold and stored at 0-5° C (Figure III-1).

2. Ribavirin from Plasma

For the extraction of ribavirin alone the use of cold 0.5N PCA was investigated. 0.5-ml samples of plasma were mixed with measured volumes of an aqueous solution of ribavirin so that the final concentration of the drug in the plasma samples was 1000, 500,

200 and 100 μg/ml. Standard solutions for comparison were generated by adding these same volumes of ribavirin solution to 0.5 ml of water. The plasma samples were extracted as previously described with 0.5N PCA. The results of HPLC analysis are shown in Figures III-7 and III-8.

In a second extraction procedure, 0.5 ml of plasma and 0.5 ml of water were mixed with a solution of ribavirin so that the final concentration of the drug in plasma and water was 10 μg/ml. The plasma sample was mixed vigorously with 1 ml of acetonitrile. The sample was centrifuged and the clear supernatant was removed and concentrated to dryness in vacuo. The residue was dissolved in 0.5 ml of water for analysis (Figure III-3).

The percent recoveries for the two procedures are shown in Table III-1. While the acetonitrile extraction method provides only a 54% recovery of ribavirin in plasma, the extract contains none of the interfering components present in the PCA extract.

3. Ribavirin Prodrug: Reduced Form from Plasma

For the extraction of the reduced form of the ribavirin prodrug, a number of solvents were investigated. A single concentration of the drug in plasma was used in this study.

A series of duplicate plasma samples were spiked with the prodrug so that the final concentration of the prodrug in plasma was 100 μg/ml. These samples were then extracted with acetonitrile, methanol, 0.5N PCA, acetone, methylene chloride, or tungstate deproteinizing reagent (TDR) using four volumes of solvent per volume of plasma.

In this study, methanol came closest to meeting our criteria. In two extraction experiments the extraction efficiencies were 85 and 95%. The reduced forms of the prodrug was stable in the extraction matrix for four days at 5 °C or lower and for at least 24 hours at room temperature. Both the reduced prodrug and its oxidized form could be detected and quantitated in a single isocratic HPLC analysis (Figure III-9). In extractions using PCA and acetonitrile, the efficiencies were acceptable but the extracts were unstable. When methylene chloride and acetone were used for extraction, the extracts were concentrated to dryness and reconstituted in the HPLC mobile phase. These solutions were also unstable. In a single experiment the stability of the reduced form of the prodrug in methanol and its instability in the HPLC buffer were confirmed by analyzing aliquots of these solutions periodically for 24 hr. The half-life of the prodrug in the buffer at 5° was less than one hour (Figure III-5). The oxidized form of the prodrug was stable in both solvents.

In further studies using methanol extracts with automated HPLC equipment compatibility problems developed. For this reason we looked again at acetonitrile as an extraction solvent and developed an efficient, relatively simple extraction procedure for the recovery of the reduced prodrug from plasma.

Using this procedure the plasma sample was extracted with four volumes of acetonitrile. The precipitated proteins were removed by centrifugation and the clear supernatant was evaporated to dryness under N_2. The residue was dissolved in 15% acetonitrile (the composition of organic solvent in the HPLC mobile phase). This procedure gave approximately 85% recovery of the drug from plasma. The recovery was independent of the concentration of the drug in plasma in the range of 100-1000 μg/ml.

C. Stability Study with the Reduced Form of the Ribavirin Prodrug

The stability of the reduced ribavirin prodrug in extracts was determined during storage at 0 °C. After overnight storage, about 10% degradation of the compound occurred. Approximately 15-20% degradation was noted after four days storage. The rate of degradation increased in samples maintained at room temperature.

These results indicated that this extraction procedure might be appropriate for pharmacokinetic studies, as long as the samples are assayed within one day of preparation.

The in vitro stability of the reduced form of the prodrug in mouse plasma and in water was determined during incubation at 5° and 37 °C. Mixtures containing 100 μg/ml of the drug and either mouse plasma or water were prepared and maintained at the indicated temperatures for 24 hr. At selected times, aliquots were removed from each mixture and assayed for the levels of the drug after acetonitrile extraction as previously described. The results are presented in Figure III-10. In plasma at 37 °C, the drug had a half-life of about 2.5 hr. At 5 °C, about 15% degradation occurred in plasma after 8 hr. The stability of the drug in water was greater but it was also temperature-dependent.

D. Metabolism of [3-^{14}C] Ribavirin to Its Nucleotides in Cells

Because both extraction and HPLC procedures for metabolism studies in mammalian cell culture systems were already in use in our laboratory, our preliminary metabolism study was conducted in cultures of mouse L1210 cells.

These cells were treated with ribavirin or [3-^{14}C] ribavirin and harvested at 1- and 2-hr time periods. The cells were collected, extracted and analyzed using the methods previously described.

Levels of ribonucleotides were determined and the quantitative effects of ribavirin on nucleotide pools are shown in Figure III-1 and summarized in Table III-2.

The metabolism of ribavirin and its effects on nucleotide pools in mouse L1210 cells are in accord with results obtained in other cells and with its demonstrated mode of action. On AS-30D hepatoma cells ribavirin caused a reduction in GTP pools with little change in ATP pools and a marked increase in pools of UTP and CTP (16). Similar reduction of GTP pools was observed in L5178YK cells (17). In rat tissues, ribavirin was metabolized to mono-, di-, and triphosphates (18). The selective reduction of GTP pools, with the subsequent build-up of IMP, is the result of inhibition of IMP dehydrogenase. Ribavirin-5'-phosphate is a potent inhibitor of this enzyme from various mammalian sources (K_i values are in the range of 0.27 - 4.2 μM) (19,20,21).

The metabolism of [3-^{14}C] ribavirin to its nucleotides was examined by collecting fractions (1 min, 2 ml) from the column eluate directly into vials and quantitatively determining radioactivity in each fraction (Figure III-2). The results of these determinations are shown in Table III-3. Radioactivity was found in the breakthrough peak, and in peaks with retention times of 1.9, 7.5, 17.5, and 34.0 min. These retention times are slightly less than those of IMP, IDP and ITP. The relative amounts of ^{14}C present in each area at 1 hr were: breakthrough, 25%; monphosphate area, 31%; diphosphate area, 20%; triphosphate area, 21%. These results were not significantly different at 2 hr. Only a relatively small amount of radioactivity was found in the acid insoluble fraction.

IV. VIROLOGY

A. Preliminary Study

This research project involves the synthesis, pharmacology, and in vivo antiviral evaluation of certain prodrug esters of ribavirin and of other antiviral drugs designed to pass the blood-brain barrier in sufficient quantities to inhibit the replication of viruses of the Togaviridae family. Ribavirin prodrugs, synthesized in this laboratory are therefore to be evaluated for therapeutic efficacy against Japanese encephalitis (JE) virus and Venezuelan equine encephalitis (VEE) virus infections in mice.

A sampie of our initial dihydropyridine conjugate of ribavirin was submitted to the Contracting Officer's Technical Representative (Dr. Michael A. Ussery) at Fort Detrick, Frederick, MD for preliminary antiviral evaluation for therapeutic efficacy in the treatment of lethal JE virus infections in mice. C57B1/6 mice (ten animals per group) were inoculated with either 10 or 1000 LD_{50} of JE virus (Peking strain) and treated with either ribavirin, ribavirin prodrug, or (for placebo-treated controls) with drug vehicle alone. The ribavirin prodrug was administered to mice by the intraperitoneal (i.p.) route at a dose level of 45 mg/kg/day once a day for nine days, beginning on Day -1 (preinfection) and ending on Day +7 (postinfection). The parent drug was administered to

mice by the same route and on the same schedule at a dose level of 50 mg/kg/day. There were no survivors at the 1000 LD_{50} virus challenge level. Among the animals challenged with 10 LD_{50} of virus, however, the data indicate that there was a 40% survival rate in the ribavirin prodrug-treated group. A 100% mortality rate was observed in both the ribavirin- and the placebo-treated groups. No significant increases in mean survival time of dying animals were observed in the treated groups when compared to virus-infected controls. These preliminary data indicated that the ribavirin prodrug crossed the blood-brain barrier and produced a significant therapeutic effect in this animal model system. The parent drug, ribavirin, was without effect in protecting mice from lethal viral encephalitis. These preliminary data are extremely encouraging and predict that highly effective prodrugs of ribavirin can be developed for the therapy of togavirus infections in man.

During this first year of the contract, much effort was expended in renovating and equipping the SRI-UAB P-3 facility, recruiting and training staff members in P-3 containment procedures, and in completing our immunization program for P-3 facility personnel. In August, 1986, building renovations were completed and the facility was certified as a Biosafety Level-3 (BL-3) containment facility. Full approval was subsequently given for appropriately immunized personnel to commence work with BL-3 level viruses. Whereas work with certain other BL-3 level viruses has been initiated, the proposed work with JE and VEE viruses in mice could not be initiated during this report period because of the failure of a number of key technical personnel to exhibit adequate serological conversion to positive neutralizing antibody titers to one or the other of these two viruses after immunization. These individuals have therefore received a booster immunization to assure that they will have adequate protection while working in the P-3 facility hot suites. During this report period our technical staff members have been trained and have become experienced in all BL-3 level operations and procedures employed in the facility.

B. Future Plans

Additional antiviral drug evaluation studies in laboratory animals will be conducted both at Fort Detrick and at Southern Research Institute (SoRI) to confirm and extend the preliminary observation of chemotherapeutic efficacy for the initial ribavirin prodrug ester against JE virus infections in mice. Experiments will also be conducted at SoRI to evaluate the therapeutic potential of this antiviral prodrug against lethal VEE infections in mice. Stocks of VEE and JE will be prepared and the viruses will be examined in several strains of mice to determine the most susceptible host animal for experimental

chemotherapy trials at SoRI. The ribavirin prodrug ester will then be tested for antiviral activity against VEE and JE viruses in vivo, utilizing all available information derived from the pharmacology studies described above. In addition, new antiviral prodrugs will be evaluated for in vivo efficacy as they become available in appropriate quantities for evaluation.

V. LITERATURE CITED

1. Sidwell, R. W., Huffman, J. H., Khare, G. P., Allen, L. B., Witkowski, J. T., and R. K. Robins. (1972) Broad-spectrum antiviral activity of virazole: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 177:705-706.

2. Huffman, J. H., Sidwell, R. W., Khare, G. P., Witkowski, J. T., Allen, L. B., and R. K. Robins. (1973) In vitro effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. Antimicrob. Agents Chemother. 3:235-241.

3. Khare, G. P., Sidwell, R. W., Witkowski, J. T., Simon, L. N., and R. K. Robins. (1973). Suppression by 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) of influenza virus-induced infections in mice. Antimicrob. Agents Chemother. 3:517-522.

4. Sidwell, R. W., Robins, R. K., and I. W. Hillyard. (1979). Ribavirin: An antiviral agent. Pharmacol. Ther. 6:123-146.

5. Smith, R. A., and W. Kirkpatrick. (1980) Ribavirin: A broad spectrum antiviral agent. Academic Press, New York, 237 pp.

6. Stephen, E. L., Jones, D. E., Peters, C. J., Eddy, G. A., Loizeaux, P. S., and P. B. Jarhling. (1980) Ribarivin tretment of toga-, arena-, and bunyavirus infections in subhuman primates and other laboratory animal species, pp. 169-183. In: Ribavirin: A broad spectrum antiviral agent (R. A. Smith and W. Kirkpatrick, eds.), Academic Press, New York.

7. Stephen, E. L. (personal communication).

8. Srivastava, P. C., and R. K. Robins. (1983) Synthesis an dantitumor activity of 2-β-D-ribofuranosylselenazole-4-carboxamide and related derivatives. J. Med. Chem. 26:445-448.

9. Kirsi, J. J., North, J. A., McKernan, P. A., Murray, B. K., Canonico, P. G., Huggins, J. W., Srivastava, P. C., and R. K. Robins. (1983) Preliminary studies of the broad spectrum antiviral activity of 2-β-D-ribofuranosylselenazole-4-carboxamide: A new antiviral agent. Antimicrob. Agents Chemother.

10. Huggins, J. W., Spears, C. T., Stiefel, J., Robins, R. K., and P. G. Canonico. (1983) Synergistic antiviral effects of ribavirin and the C-nucleoside analogues tiazafurin and selenazole against togaviruses and arenaviruses. Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., p. 108.

11. Bodor, N., and H. H. Farag. (1983) A redox chemical drug-delivery system and its use for brain-specific delivery of phenethylamine. J. Med. Chem., 26:313-318.

12. Bodor, N., and H. H. Farag. (1983) Brain-specific delivery of dopamine with a dihydropyridine pyridinum salt type redox delivery system. J. Med. Chem., 26:528-534.

13. Rand, K. H., Bodor, N., El Koussi, A. A., Raad, I., Miyake, A., Houck, H., and N. Gildersleeve. (1986). Potential treatment of herpes simplex virus encephalitis by brain-specific delivery of trifluorothymidine using a dihydropyridine pyridinium salt type redox delivery system. J. Med. Virol. 20:1-8.

14. Venkatraghavan, V., Shek, E., Perchalski, R., and N. Bodor. (1986) Brain-specific chemical delivery system for acyclovir. The Pharmacologist, 28:145 (Abstr. No. 296).

15. Rose, L. M. and R. W. Brockman. (1977). Analysis by high-pressure liquid chromatography of 9-β-D-arabinofuranosyladenine 5'-triphosphate levels in murine leukemia cells. J. Chromat., 133:335-343.

16. Kaiser, W. A., Herrman, B., and D. O. R. Keppler. (1980). Selective guanosine phosphate deficiency in hepatoma cells induced by inhibitors of IMP dehydrogenase. Hoppe-Seyler's β-Physiol. Chem., 361:1503-1510.

17. Muller, W. E., Maidhof, A., Taschner, H., and R. K. Zahn. (1977). Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide): a cytostatic agent. Biochem. Pharmacol., 26:1701-1705.

18. Miller, J. P., Kigwana, L. J., Streeter, D. G., Robins, R. K. Simon, L. N., and John Roboz. (1977). The relationship between the metabolism of ribavirin and its proposed mechanism of action. N. Y. Acad. Sci., 284:211-229.

19. Streeter, D. G., Witkowski, J. T., Gyaneshwar, P. K., Sidwell, R. W., Bauer, R. J., Robins, R. K., and Lionel N. Simon. (1973). Mechanism of action of 1-β-D-ribafuranosyl-1,2,4-triazole-3-carboxamide (virazole), 9 new broad-spectrum antiviral agent. Proc. Nat. Acad. Sci. USA, 70:1174-1178.

20. Fuertes, M., Witkowski, J. T., Streeter, D. G., and R. K. Robins. (1974). Synthesis and enzymatic activity of 1,2,4-triazole-3-carboxamide-6'deoxyhomoribonucleoside-6-phosphonic acid and related compounds. J. Med. Chem., 17:642-645.

21. Gebeyehu, G., Marquez, V. E., Van Cott, A., Cooney, D., Kelley, J. A., Hiremagalur, N. J., Gurprett, S. A., Dion, R. L., Wilson, Y. A., and D. H. Johns. (1985). Ribavirin, tiazofurin, and selenazofurin: mononucleotides and nicotinamide adenine dinucleotide analogues. Synthesis, structure, and interactions with IMP dehydrogenase. J. Med. Chem., 28:99-105.

VI. ACKNOWLEDGMENTS

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APPENDIX A
(Tables)

Table III-1

A Comparison of the Percent Recoveries for Two Extraction Methods: 0.5N PCA and Acetonitrile

0.5N PCA Extraction

Sample No.	Integrator Response* H_2O + Ribavirin	Plasma + Ribavirin	% Recovery
1	(Blank)	(Blank)	
2	0.22×10^7	0.26×10^7	120 (!)
3	0.46×10^7	0.27×10^7	59
4	1.11×10^7	0.78×10^7	71
5	2.10×10^7	1.06×10^7	50

CH_3CN Extraction

Sample No.	H_2O + Ribavirin	Plasma + Ribavirin	% Recovery
1	1.12×10^7	0.60×10^7	54

* Integrator responses were influenced by the elution of a peak preceding the peak of interest.

Table III-2

Nucleotide Pools in L1210 Cells Treated with Ribavirin

Treatment	Concentration (μm)	Time[2] (hr)	Nanomoles of Nucleotide per 10^9 Cells[3]									
			AMP	ADP	ATP	Σ	IMP	GDP	GTP	Σ	CTP	UTP
Control			250	772	1004	2026	118	256	300	556	120	244
[3-^{14}C] Ribavirin	15[1]	1	362	526	565	1453	860	164	200	364	137	340
[3-^{14}C] Ribavirin	15[1]	2	297	549	595	1441	836	158	147	305	149	348
Ribavirin	102	1	300	395	430	1125	915	102	77	179	147	374
Ribavirin	102	2	380	423	716	1519	832	83	100	183	230	654
			% of Control									
[3-^{14}C] Ribavirin	15[1]	1	145	68	56	72	729	64	67	65	114	139
[3-^{14}C] Ribavirin	15[1]	2	119	71	59	71	708	62	49	55	124	143
Ribavirin	102	1	120	51	43	56	775	40	26	32	123	153
Ribavirin	102	2	152	55	71	75	705	32	33	33	192	268

[1] 0.5 μCi/ml

[2] Time of incubation of cells with drug prior to harvest and cold PCA extraction.

[3] Anion exchange HPLC analysis.

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Table III-3

Metabolism of [3-^{14}C] Ribavirin to Mono-, Di- and Triphosphates in L1210 Cells

Inhibitor	Retention Time (min)	HPLC Analysis of Perchloric Acid-Soluble Extracts from L1210 Cells[1] pCi/10^7 cells 1 hr	pCi/10^7 cells 2 hr	% of Total 1 hr	% of Total 2 hr
[3-^{14}C] Ribavirin	1.9	2458	9492	25	20
[3-^{14}C] Ribavirin	7.5	3046	3640	31	26
[3-^{14}C] Ribavirin	17.5	1946	2774	20	19
[3-^{14}C] Ribavirin	34.0	2036	3096	21	22
Acid-insoluble fraction		236	398		

[1] Fractions (2 ml) from anion-exchange HPLC analysis of extracts from L1210 cells were collected for quantitative determination of radioactivity.

APPENDIX B
(Figures)

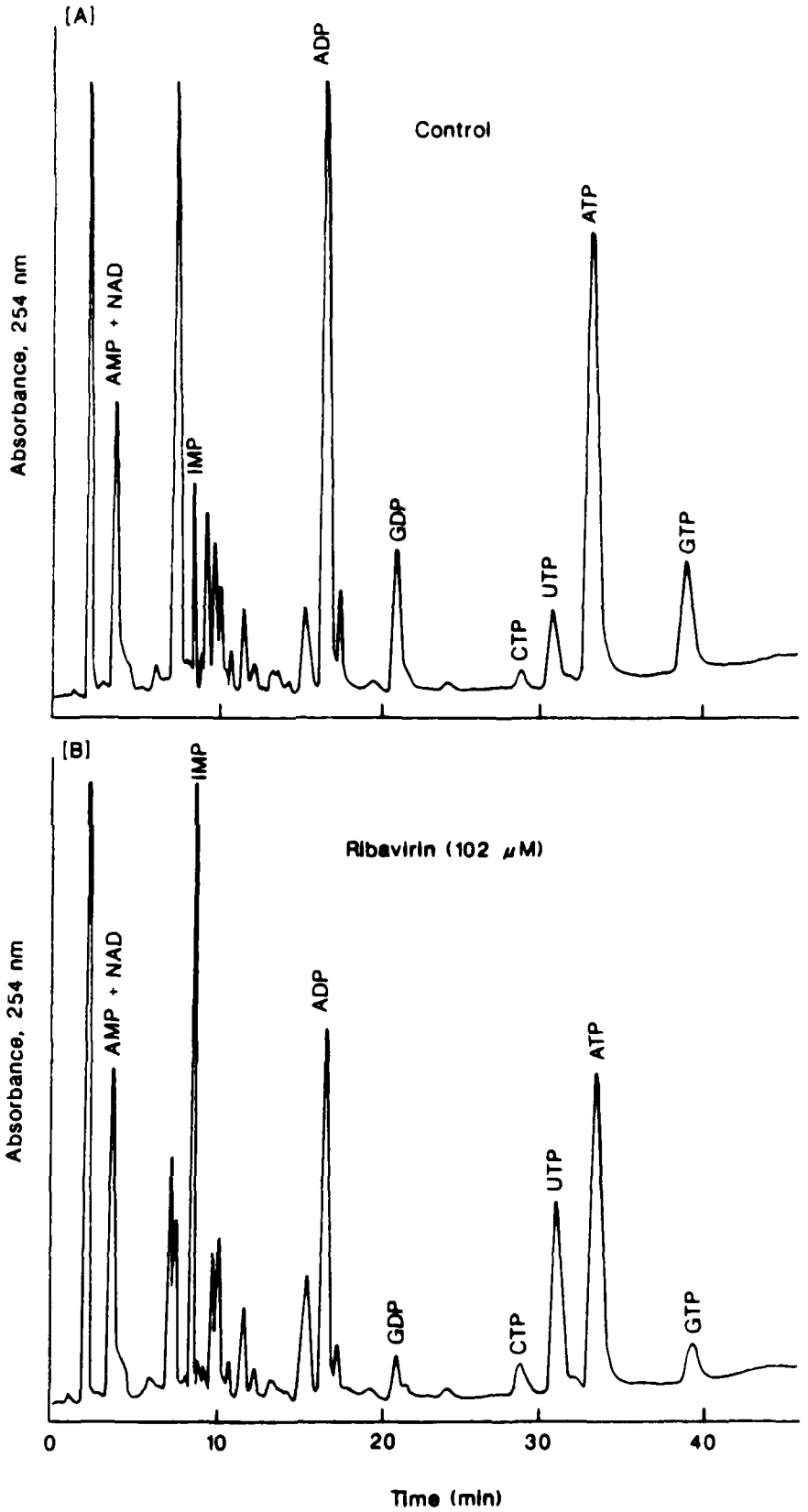

Figure III-1. The effect of Ribavirin on ribonucleotide pools in L1210 cells. Cold PCA extracts of cells were analyzed by anion-exchange HPLC as dexcribed in the text. [A] Control cells; [B] Cells treated with Ribavirin.

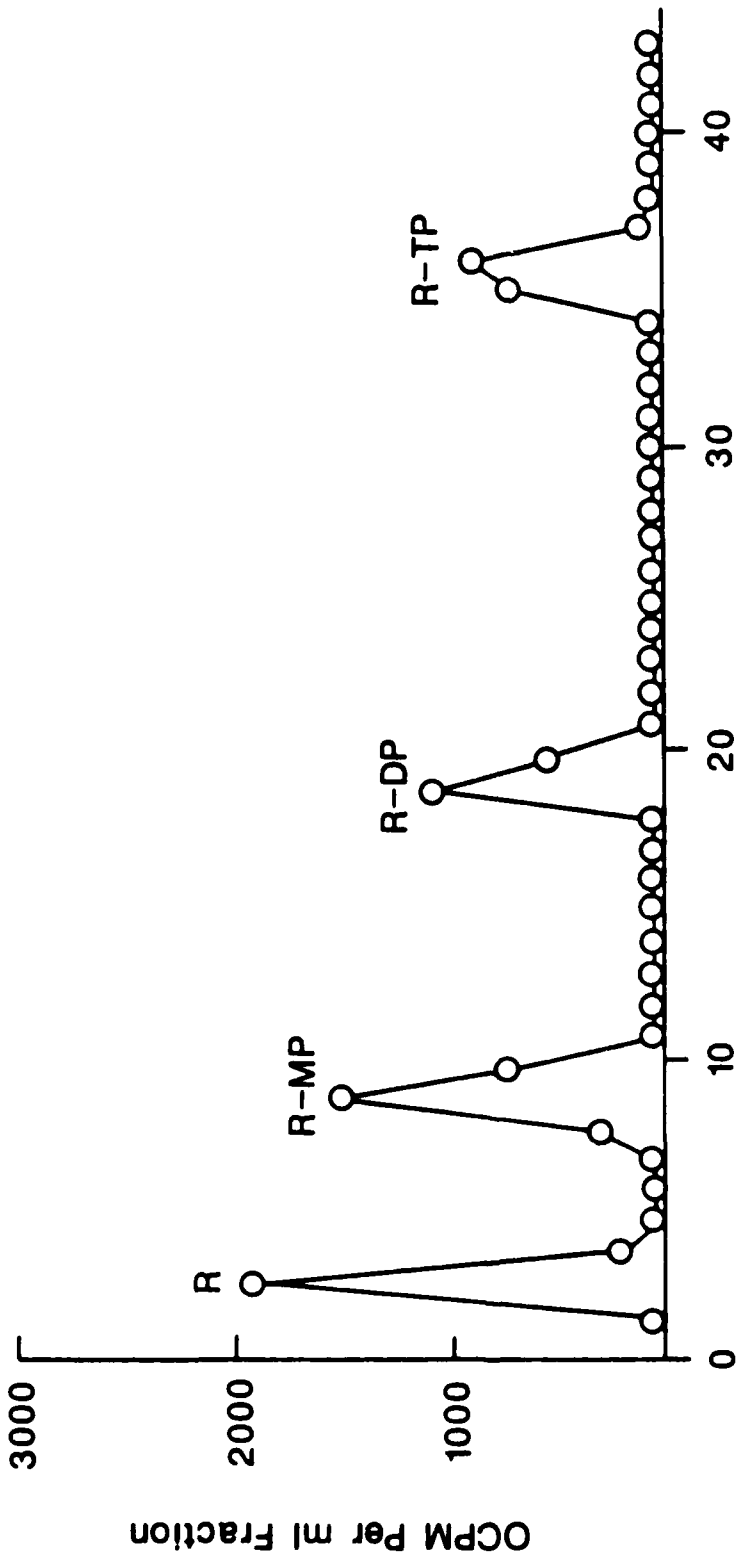

Figure III-2. Metabolism of [3-^{14}C]Ribavirin (R) to mono-, di and triphosphates in L1210 cells. Fractions (2 ml) from anion-exchange HPLC analysis of cold PCA extracts were collected for quantitative determination of radioactivity.

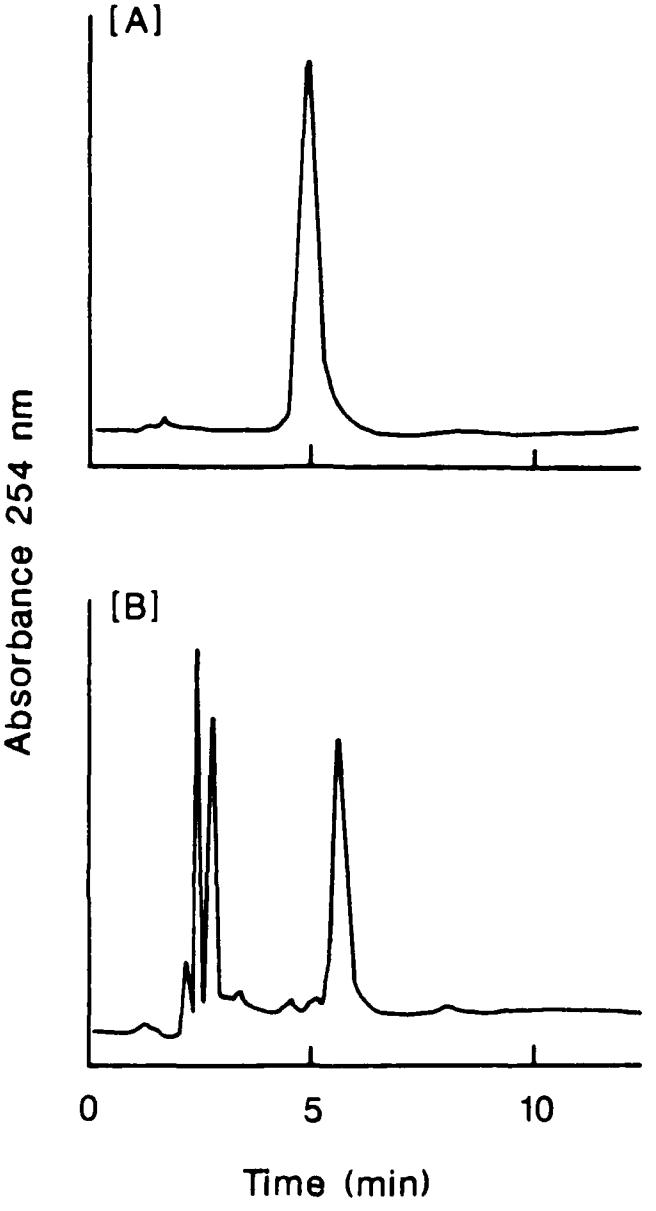

Figure III-3. Reversed phase HPLC analyses of a ribavirin standard and of an acetonitrile extract of plasma to which ribavirin was added. The concentration of ribavirin in each sample was the same (500 μg/ml) so that a direct measurement of percent recovery could be made. A, chromatogram of ribavirin standard; B, chromatogram of the acetonitrile extract of plasma plus ribavirin.

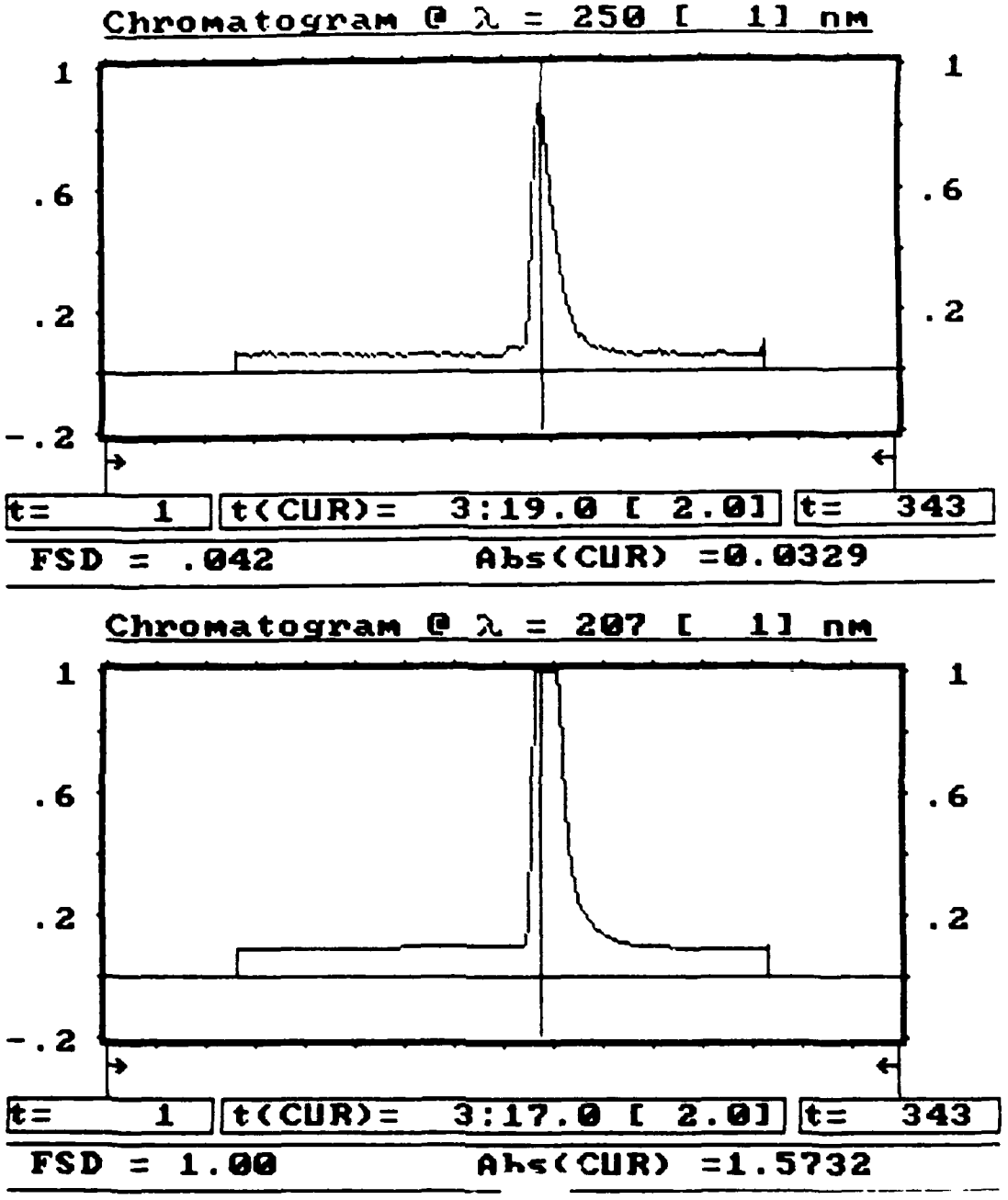

Figure III-4. A comparison of the UV absorbance of ribavirin at 250 nm and at 207 nm, its UV maximum.

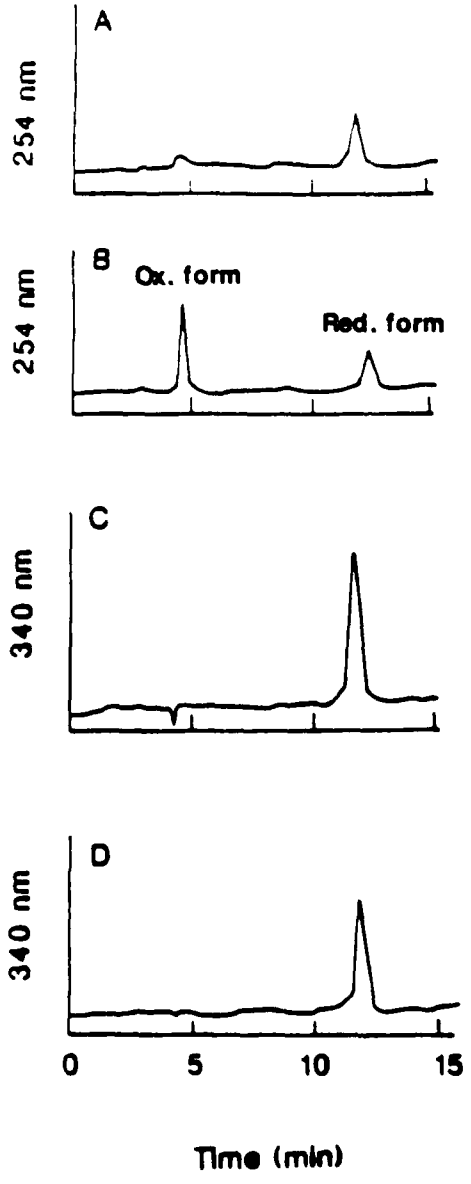

Figure III-5. Reversed phase HPLC analyses of aliquots of the prodrug in 25 mM $NH_4H_2PO_4$ (pH 4.5): CH_3CN (85:15,V/V).
A, solution at T_0 monitored at 254 nm;
B, solution at T_{50} monitored at 254 nm;
C, solution at T_0 monitored at 340 nm;
D, solution at T_{50} monitored at 340 nm.

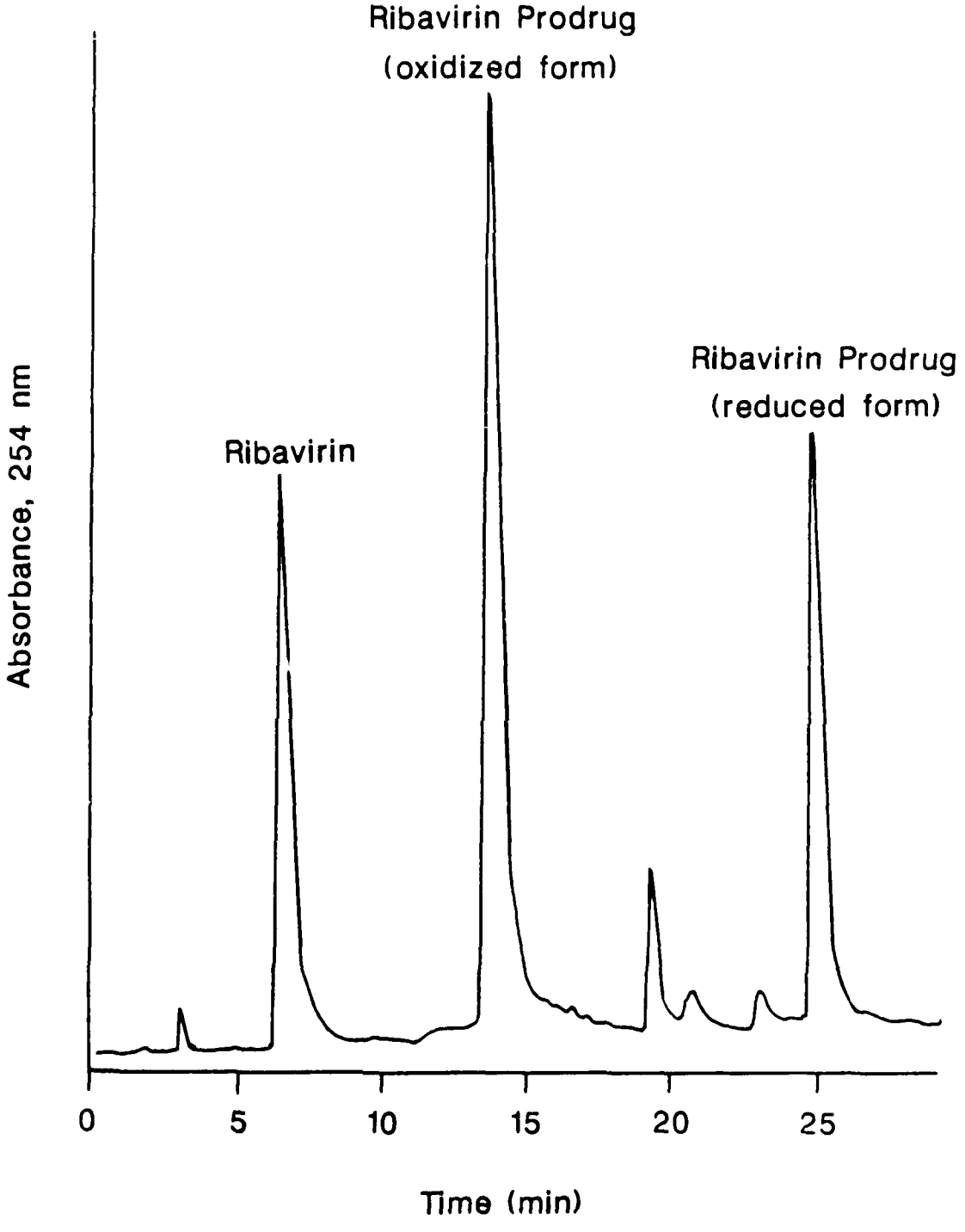

Figure III-6. Separation of Ribavirin and the oxidized and reduced forms of the Prodrug by reverse phase chromatography as described in the text.

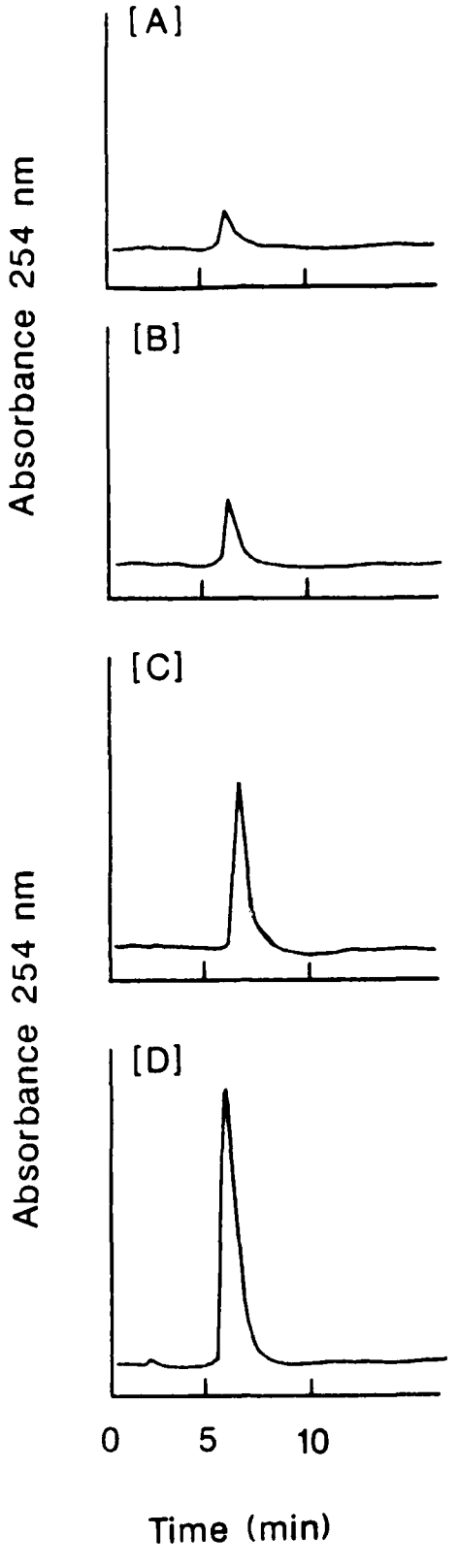

Figure III-7. Reversed phase HPLC analyses of 100 µl aliquots of ribavirin standards. A, 100 µg/ml; B, 200 µg/ml; C, 500 µg/ml; D, 1000 µg/ml.

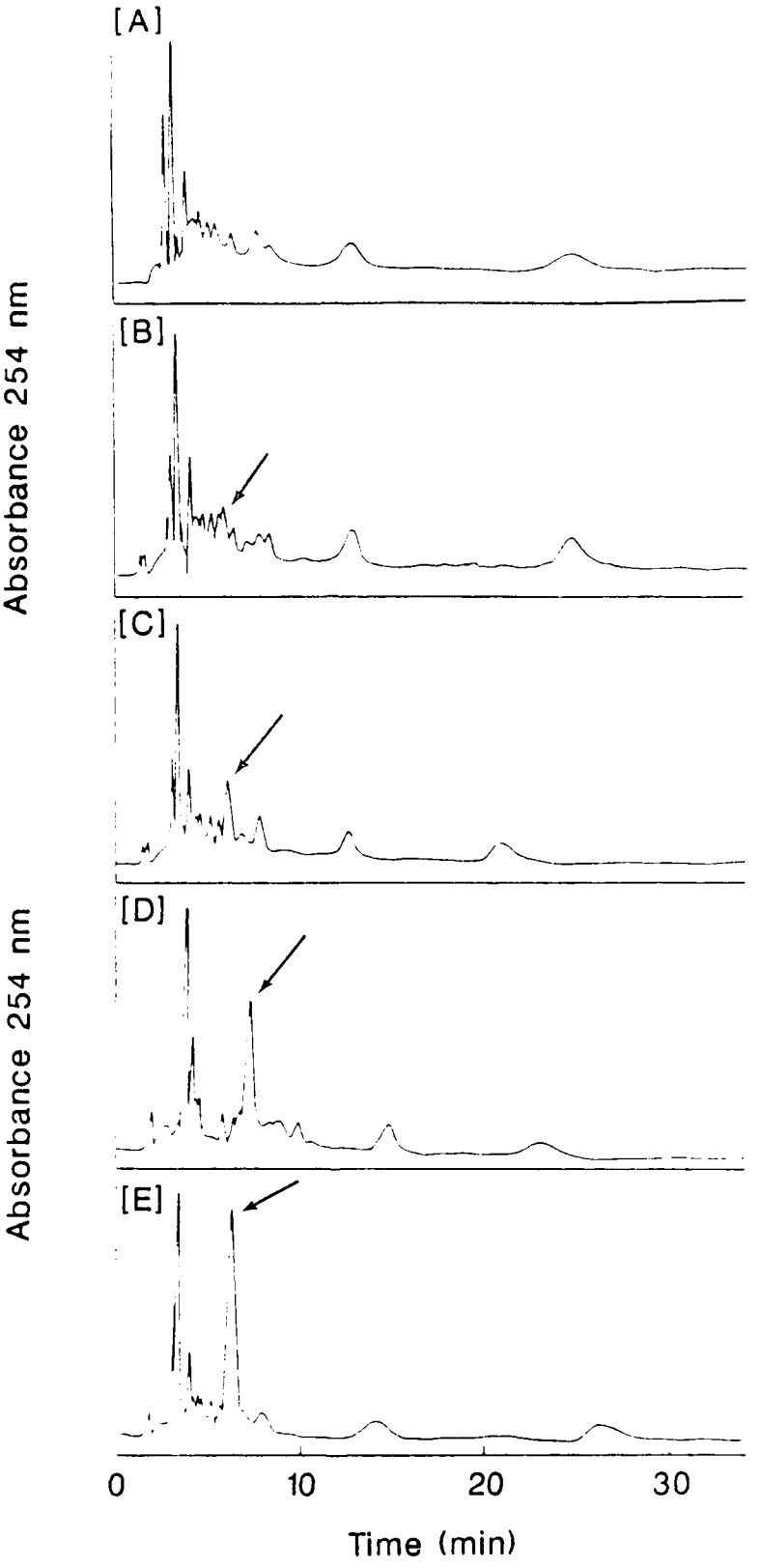

Figure III-8. Reversed phase HPLC analyses of 0.5N PCA extracts of plasma to which ribavirin had been added to final concentrations of 100, 200, 500, and 1000 μg/ml. A, extract of plasma; B, extract of plasma plus ribavirin at 100 μg/ml; C, extract of plasma plus ribavirin at 200 μg/ml; D, extract of plasma plus ribavirin at 500 μg/ml; E, extract of plasma plus ribavirin at 1000 μg/ml.

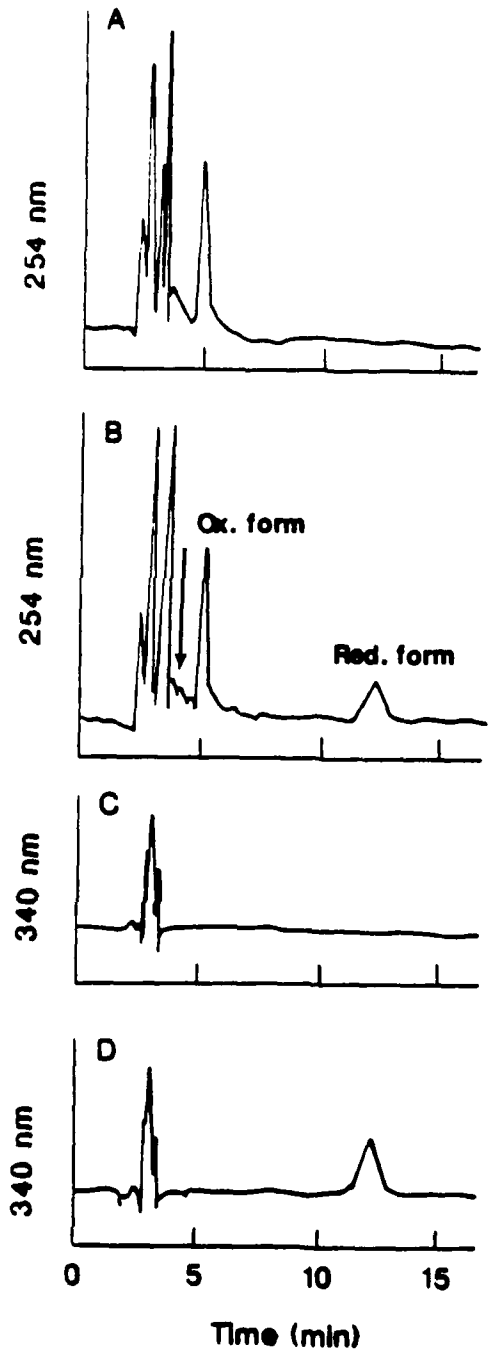

Figure III-9. Reversed phase HPLC analyses of methanol extracts of plasma spiked with the N-methyldihydropyridinium derivative of ribavirin (prodrug) at 100 μg/ml.

A, extract of plasma monitored at 254 nm;
B, extract of plasma plus prodrug monitored at 254 nm;
C, extract of plasma monitored at 340 nm;
D, extract of plasma plus prodrug monitored at 340 nm.

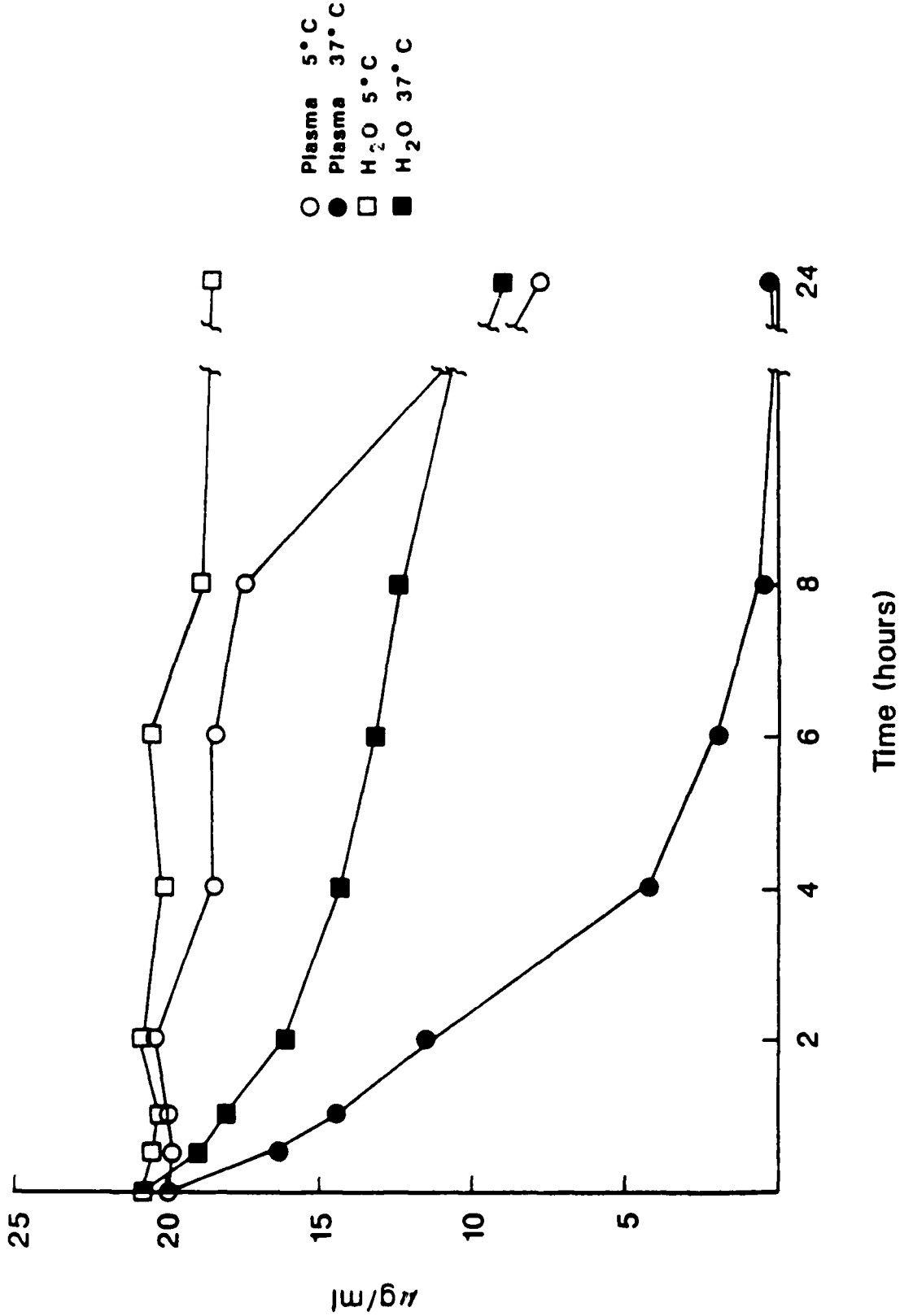

Figure III-10. Stability of SRI 6711 in plasma and H_2O. Mixtures of SRI 6711 in plasma or water, at either 5 °C or 37 °C, were assayed at the times indicated. Value on the y axis represents μg SRI 6711/ml of the extract assayed.

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